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<b>(21) International Application Number:</b> PCT/US96/20672 <b>(22) International Filing Date:</b> 27 December 1996 (27.12.96) <b>(30) Priority Data:</b> 08/580,752 29 December 1995 (29.12.95) US <b>(71)(72) Applicants and Inventors:</b> PIPER, Robert, C. [US/US]; Flow Incorporated, 6127 S.W. Corbett, Portland, OR 97201 (US). MAKLER, Michael, T. [US/US]; Flow Incorporated, 6127 S.W. Corbett, Portland, OR 97201 (US). <b>(74) Agent:</b> NEBEL, Heidi, Sease; Zarley, McKee, Thomte, Voorhees & Sease, Suite 3200, 801 Grand Avenue, Des Moines, IA 50309-2721 (US).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> MONOCLONAL ANTIBODIES AND IMMUNO-CAPTURE METHOD FOR QUANTITATION AND SPECIATION OF MALARIA PARASITES		
<b>(57) Abstract</b> <p>An improved method for the detection and quantitation of malarial infection is defined. A series of monoclonal antibodies have been developed which can capture the parasitic enzyme lactate dehydrogenate and are specific for <i>Plasmodium</i> LDH without binding human LDH. The activities assays and the result is a pLDH assay which is 10-100 fold more sensitive than previous disclosed assay methods. The invention further discloses monoclonal antibodies capable of differentiating LDH isoforms from different species of plasmodium that infect humans.</p>		

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TITLE: MONOCLONAL ANTIBODIES AND IMMUNO-CAPTURE METHOD  
FOR QUANTITATION AND SPECIATION OF MALARIA  
PARASITES

5 BACKGROUND OF THE INVENTION

Malaria, a protozoan disease transmitted by the Anopheles mosquito, affects millions of people each year and has been characterized as "the most important of all infectious diseases" (Sir Macfarlane Burnet). There are over 200 million cases of malaria annually with the number of clinical cases exceeding 120 million. Approximately 2 million of those cases result in death, the majority of which are infants and young children.

Malaria remains endemic in many countries including Africa, Central America, the Caribbean Islands, South America, Europe, Asia, Indian sub-continent, Eastern Asia and Oceania. The disease is characterized by fever, rigors, anemia, splenomegaly, and chronic relapsing course.

In humans, the disease can be caused by four species of protozoa: *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale*, and *Plasmodium falciparum*. These *Plasmodia* go through a number of morphological changes with multiplication of the organisms in man and in insect vectors. Man is infected by the saliva of a female anopheline mosquito that contains the infective sporozoite form. This sporozoite then enter the liver cells where they multiply. After approximately seven to ten days, multiple small forms break out of the liver cells into the blood and subsequently enter red blood cells. The dormancy of the infection in the liver cells explain relapses in malaria caused by *Plasmodium vivax* and *Plasmodium ovale*. While in the red blood cells, the organisms produce multiple infective merozoites. Upon bursting of the erythrocyte, this form can enter new red blood cells. The duration of the cycle is approximately two days for *P. vivax*, *P. ovale*, and *P. falciparum* and three days for *P. malariae*. This phase of the cycle presents clinically as erythrocytic febrile attacks. Some of these merozoites become sexual forms or gametocytes which when consumed by mosquitoes complete the life cycle.

Most cases of malaria reported in the United States are found in travelers recently exposed to mosquitoes in endemic areas. Additionally, transmission may occur in drug addicts sharing needles and syringes or by blood transfusions. Rarely, infection is caused congenitally or by mosquito transmission from imported cases. Delays often occur in the recognition and treatment of malaria because American physicians fail to consider this latter mode of transmission in their differential diagnosis. Further, physicians also neglect to advise travelers of a need to receive chemoprophylaxis for malaria when traveling to endemic areas.

In addition, over the last twenty years there has been an increasing need to develop new approaches to prevent the spread of malaria. The spread of malaria has been exacerbated by the parasites' increased resistance to standard antimalarials such as chloroquine, mefloquine, and quinine. There is therefore a necessary and real incentive to develop, refine and further format a diagnostic assay for malaria to rapidly and accurately diagnose malaria cases which can also detect drug resistance.

Prior art diagnostic tests for malaria involve examination of an appropriately stained blood film. Morphological differences among the plasmodial species infecting humans allows identification in blood smears. Usually, fingertip blood is smeared on a glass slide and stained with Wright's, Giemsa's, or Field stain. A disadvantage to this type of diagnostic test is that thick smears usually contain other unwanted artifacts which can result in inaccurate diagnoses.

Another diagnostic procedure involves the use of thin blood smears. While thin smears are easier to examine, the scarceness of cells in a thin smear increases the likelihood that a diagnosis will be missed. Additionally, because the intensity of parasitemia varies greatly from hour to hour, frequent repeated examinations of thin blood smears may be required which may be prohibitively labor intensive and expensive, especially in endemic areas.

It is known that antiplasmodial antibodies are produced in animals during a malarial infection. For example, using hybridoma technology, Taylor, D.W. et al., *Infection Immunity*,

32(2):563-570 (1981), identified monoclonal antibodies (MAbs) to *Plasmodium voelii*, a rodent malarial parasite. These MAbs identified stage specific, species specific, and cross-reactive antigens. The antigens were found on the surface or within the cytoplasm of the parasite, but not on the surface of erythrocytes from infected animals.

Malarial antigens are also known to appear in the serum of humans infected with *P. falciparum*. McGregor, I.A., et al., *Lancet* 1:881-884 (1968). The antigens may be secreted from infected erythrocytes for release during erythrocyte rupture and reinvasion. Winchell, E.J., et al., *Molecular Biochemistry and Parasitology* 10:287-296 (1984). U.S. Pat. No. 5,001,225 issued to Taylor on March 19, 1991 discloses a hybridoma cell line which secretes a monoclonal antibody which binds to a protein present in *Plasmodium* which may be used for immunodiagnostic and immunotherapeutic treatment of malarial diseases of man and animals.

It has previously been reported that parasitic LDH (pLDH) can be used as a means of detecting the presence of *P. falciparum*. Makler et al. 1993, "Measurement of the Lactate Dehydrogenase Activity of *Plasmodium falciparum* as an Assessment of Parasitemia", *Am. J. Trop. Med. Hyg.*, 48(2), pp. 205-210. LDH is a metabolic enzyme found within the parasite's cytoplasm and each parasite contains 1-2 nUnits of LDH activity. This method comprises an enzyme assay which measures the ability of *Plasmodium* LDH (pLDH) to rapidly use 3-acetyl pyridine NAD as a coenzyme in the reaction leading to the formation of pyruvate from lactate. Further, Kaushal, et. al. demonstrated that polyclonal immune monkey serum raised against schizonts of *Plasmodium knowlesi* (H-strain) showed the presence of antibodies to LDH of *P. knowlesi*. Kaushal, D.C. et al., "Antibodies to Lactate Dehydrogenase of *Plasmodium Knowlesi* are Specific to *Plasmodium* Species", *Immunological Investigations*, 17:507-516 (1988). Thus far, however, the antigenic properties of pLDH have not been addressed for their potential role in the diagnosis of malaria in humans.

It is therefore an objective of the present invention to provide a novel method for the quantitation and/or speciation of malarial parasites.

5 It is another objective of the present invention to detect Plasmodium lactate dehydrogenase, a marker which can be used to indicate the presence of active, malaria causing parasites in humans.

10 It is a further objective of the present invention to provide monoclonal antibodies which detect the presence of plasmodium LDH which can be used to assay for its presence for purposes of diagnosing malaria.

15 It is yet another objective of the present invention to provide a method of distinguishing pLDH using a series of monoclonal antibodies which may be used in both wet and dry assays.

It is still another objective of the present invention to provide a method of diagnosing malaria which is rapid, sensitive, specific, quantitative, and easy to perform..

20 These and other objectives of the invention will become apparent from the foregoing description.

#### **SUMMARY OF THE INVENTION**

This invention discloses an improved method for the detection and quantitation of malarial infection. A series of  
25 monoclonal antibodies has been developed that can capture an active metabolic enzyme, specifically Plasmodium lactate dehydrogenase (pLDH). The monoclonal antibodies are specific for Plasmodium LDH and do not cross-react with LDH isoforms present in whole blood of human, bovine, murine, avian, or  
30 other primate sources of LDH. The activity of the captured pLDH may then be assayed using standard assay procedures which are well known to those of ordinary skill in the art. Preferably, the pLDH is assayed using the LDH substrate lactate and 3-acetyl pyridine nicotinamide adenine dinucleotide as  
35 specified in U.S. Patent 5,124,141 dated June 23, 1992, the disclosure of which is hereby incorporated by reference. This novel assay method has increased the specificity of the pLDH

assay as recorded in Patent No. 5,124,141 by ten to one hundred fold.

The present invention also encompasses the development of a panel of monoclonal antibodies capable of differentiating LDH isoforms from different species of *Plasmodium* that infect humans. These species include *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae*. A subset within this panel of monoclonal antibodies can capture pLDH from the human-specific *Plasmodium* species in the fully active form. Binding competition studies used to epitope map the antibodies show that the monoclonal antibodies bind to sites distinct from one another.

The present invention further involves a test based upon the specificity of the monoclonal antibodies which may be used to differentiate between different species of malaria-causing parasites in humans as well as *Plasmodium* from murine, avian, and primate sources. Since correct speciation is a critical feature for diagnostic and epidemiological studies of malaria, this new set of tests is extremely useful for the efficient and timely diagnosis of malaria. These tests are based upon the fact that the monoclonal antibodies directed to pLDH are able to capture these active parasitic enzymes from the sample, whether this be hemolyzed red cells, plasma, serum, saliva, or other body fluids. During the capture process, the catalytic site of the pLDH remains enzymatically active, thereby making it possible to detect the captured enzyme by a previously developed and patented diagnostic reagent. The invention includes several enzyme-immuno assay formats which are capable of capturing pLDH from different *Plasmodium* species.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph demonstrating the column chromatography of pLDH using Cibacron blue sepharose chromatography from culture lysates. pLDH activity (APAD) can be separated from human red blood cell LDH (1st NAD peak) because pLDH binds tightly to Cibacron blue (Column profile).

Figure 2 is an agarose electrophoresis gel in which recombinant pLDH has been stained with NAD<sup>+</sup>, NBT and PES.

Figure 3 are microtiter plates which have been coated with the MAbs: 6C9a, 6C9b, 17E4, 19G7, and 7G9 which demonstrate that the plates are capable of capturing enzymatically active pLDH from blood samples.

5 Figure 4 is a graph showing the measurement of specific activities (mOD/min) of 7 samples of *P. falciparum* and 3 samples of *P. vivax*.

Figure 5 is a graph showing the quantitation of the ICpLDH assay demonstrating the parasitic activity (mOD/ml) plotted as  
10 a function of % parasitemia.

Figure 6 shows microtiter wells coated with either the 19G7 monoclonal antibody (pan-specific) or the 17E4 monoclonal antibody (*P. falciparum* specific). The wells were washed 2 times with PBS and incubated with APAD containing NBT and  
15 Diaphorase. Either 100  $\mu$ l or 5  $\mu$ l of blood sample were incubated in a coated microtiter well for 30 minutes.

Figure 7 is a graph demonstrating the activity of pLDH as tested in the presence of the anti-pLDH antibodies 6C9a, 7G9, 17E4, and 19G7. A solution of recombinant pLDH was incubated  
20 for five minutes with the indicated concentration of antibody ( $\mu$ g/ml) prior to assay with APAD. Only the 6C9a antibody was inhibitory to the enzyme activity.

Figure 8 visually depicts the activity of the indicated *Plasmodium* species when incubated in microtiter wells coated  
25 with the indicated antibody. The 6C9a antibody recognized all *Plasmodium* LDH isoforms.

#### DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

The presence of antibodies to key metabolic enzymes of  
30 parasites has been demonstrated in the infected host during certain parasitic infections, but their role has not been critically assessed. *Plasmodium* lactate dehydrogenase (pLDH), the terminal enzyme of the glycolytic pathway, has been demonstrated in several malarial parasites. This enzyme plays  
35 an important role in the regulation of intracellular redox state and thus affects the energy metabolism of these parasites. Although considerable work has been done to study



the kinetic and electrophoretic behavior of *Plasmodium* LDH, little has been demonstrated about its antigenic nature.

The present invention is directly related to the antigenic nature of pLDH in the development of monoclonal antibodies and an immunocapture method for quantifying and speciating malarial parasites. This invention is thus useful in the diagnosis and detection of malaria in humans caused by the human malarial parasites *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae*. The assay system is based upon the quantitation of previously captured pLDH.

This parasite enzyme assay can detect pLDH produced by all four species of *Plasmodium* that infect humans, thus making the specific and sensitive measure of pLDH a useful tool for the diagnosis of all forms of malaria. Because the measure of pLDH is quantitative and reflects the presence of viable parasites, such a measure is also useful to quickly test the efficacy of drug treatment for a particular malarial infection, to determine the presence of drug resistance, and to also detect the presence of parasites in blood products. The invention sets forth a unique method which: (1) enhances the sensitivity and specificity of the measurement of pLDH; (2) relates the levels of pLDH whole blood to the percent parasitemia and the severity of the disease; (3) formats an easy to perform, simple to interpret, inexpensive, and quantitative diagnostic test for malaria using whole blood, lysed red blood cells, and plasma-serum; and (4) modifies the immuno-enzyme assay to reformat an in-vitro culture and sensitivity assay to evaluate multiple drug resistance under field conditions.

As such, according to the invention, monoclonal antibodies that specifically react with pLDH have been prepared. Experiments with these MAbs have shown that they effectively quantify and identify the particular *Plasmodium* species which the host is infected with. The MAbs of the invention were obtained using methods standard in the art for production of such antibodies.

Numerous studies in the literature have studied the characteristics of *Plasmodium* LDH. However, the prior art has not yet disclosed a method for producing monoclonal antibodies

directed towards specific *Plasmodium* species which cause malaria in humans nor do they identify a method of specifically identifying the particular *Plasmodium* species causing the infection.

5       The invention thus embodies a method for detection of pLDH, a marker of active malaria causing *Plasmodium* parasites, and a method of diagnosis, therapy, or study of the same by assaying for the presence of pLDH. The presence of the pLDH, may be ascertained by collecting a biological sample which may  
10   include serum, plasma, urine, saliva, or other body fluids. One method involves exploitation of the immunogenicity of the pLDH by developing monoclonal antibodies and immunological quantification. Conventional immunoassays are well known and readily ascertained by those of ordinary skill in the art. A  
15   preferred immunoassay uses a specialized detection reagent consisting of 3-acetyl pyridine adenine dinucleotide (APAD) in combination with a selected substrate, such as a lactate salt or lactic acid, and a buffer, as described in U.S. Pat. No. 5,124,141 incorporated herein by reference. The combination of  
20   the reagent with a sample of parasite-infected blood catalyzes oxidation of the substrate and simultaneously reduces the APAD. The host LDH has no influence on the APAD.

      In the preferred assay, reduced APAD may be detected in numerous ways. For example, the reduced material may be  
25   spectrophotometrically detected by observing its characteristic absorption peak at about 363-365 nm, which is distinctive from the absorption spectra of a sample containing unreduced APAD. It is also possible to colorimetrically detect the reduced APAD using a series of chromogens. The chromogens (e.g. tetrazolium  
30   salts) react in the presence of the reduced APAD to generate a colored product which is visually detectable. Finally, the reduced APAD is fluorescent, and may be detected using an appropriately configured fluorescent lamp system or fluorometer. Regardless of which detecting method is used, the  
35   present invention enables the rapid and accurate detection of malaria-infected blood in a manner not previously known in the art.

Monoclonal antibodies are prepared by recovering spleen cells from immunized animals and immortalizing the cells in conventional fashion, e.g., by fusion with myeloma cells or by Epstein-Barr virus transformation in screening for clones expressing the desired antibody. The hybridoma technique described originally by Koehler and Milstein, *Eur. J. Immunol.*, 6:511 (1976) and also described by Hammerling et al., in "Monoclonal Antibodies and T-Cell Hybridomas", Elsevier, New York, pp. 563-681 (1981) have been widely applied to produce hybrid cell lines that secrete high levels of monoclonal antibodies against many specific antigens. The hybrid cell lines can be maintained *in vitro* in cell culture media. The cell lines producing the antibodies can be selected and/or maintained in a medium containing hypoxanthine-aminopterin thymidine (HAT). In fact, once the hybridoma cell line is established, it can be maintained on a variety of nutritionally adequate media. Moreover, the hybrid cells lines can be stored and preserved in any number of conventional ways, including freezing and storage under liquid nitrogen. Frozen cell lines can be revived and cultured indefinitely with resumed synthesis and secretion of monoclonal antibody.

The secreted antibody is recovered from tissue culture supernatant or ascites fluid by conventional methods such as immune precipitation, ion exchange chromatography, affinity chromatography such as protein A/protein G column chromatography, or the like. The antibodies described herein are also recovered from hybridoma cell cultures by conventional methods such as precipitation with 50% ammonium sulfate. The purified antibodies can then be sterile filtered.

The term "monoclonal antibody" as used herein refers to any antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against

different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they are synthesized by the hybridoma culture, uncontaminated by other immunoglobulins.

The monoclonal antibodies herein also include hybrid and recombinant antibodies produced by splicing a variable (including hypervariable) domain of an anti-pLDH antibody with a constant domain (e.g. "humanized" antibodies), or a light chain with a heavy chain, or a chain from one species with a chain from another species, or fusions with heterologous proteins, regardless of species of origin or immunoglobulin class or subclass designation, as well as antibodies fragment (e.g., Fab, F(ab')<sub>2</sub> and Fv), so long as they exhibit the desired biological activity. (See e.g. Cabilly et al. U.S. Patent No. 4,816,567; Mage and Lamoyi, "Monoclonal Antibody Production Technique and Applications", pp. 79-97 (Marcel Dekker, Inc., New York, 1987)).

Thus, the modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogenous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method described by Koehler and Milstein, supra, or may be made by recombinant DNA methods (Cabilly, et al. supra).

Anti-pLDH antibodies are useful in diagnostic assays for pLDH expression in specific cells or tissues wherein the antibodies are labeled as described below and are immobilized on an insoluble matrix. Anti-pLDH antibodies also are useful for the affinity purification of the pLDH from recombinant cell culture or natural sources. The anti-pLDH antibodies that do not detectably cross react with other pLDH can be used to purify each pLDH free from other homologous receptors.

Suitable diagnostic assays for the pLDH are well known per se. For example, a biological sample may be assayed for pLDH by obtaining the sample from a desired source, admixing the sample with anti-pLDH antibody to allow the antibody to form

antibody/pLDH complex with any pLDH present in the mixture and detecting any antibody/pLDH complex present in the mixture. The biological sample may be prepared for assay by methods known in the art which are suitable for the particular sample.

- 5 The methods of admixing the sample with antibodies and the methods of detecting antibodies/pLDH complex are chosen according to the type of assay used. Such assays include competitive and sandwich assays, and steric inhibition assays. Competitive and sandwich methods employ a phase-separation step  
10 as an integral part of the method while steric inhibition assays are conducted in a single reaction mixture.

- Analytical methods for the pLDH all use one or more of the following reagents: labeled pLDH analog, immobilized pLDH analog, labels anti-pLDH antibody, immobilized anti-pLDH  
15 antibody and steric conjugate.

- The label used is any detectable functionality that does not interfere with the binding of pLDH and anti-pLDH antibody. Numerous labels are known for use in immunoassay, examples including moieties that may be detected directly, such as  
20 fluorochrome, chemiluminescent, and radioactive labels, as well as moieties such as enzymes, that must be reacted or derivatized to be detected. Examples of such labels include the radioisotopes  $^{32}\text{P}$ ,  $^{14}\text{C}$ ,  $^{125}\text{I}$ ,  $^3\text{H}$ , and  $^{131}\text{I}$ , fluorophores such as rare earth chelates or fluorescein and its derivatives,  
25 rhodamine and its derivatives, dansyn, umbelliferone, luciferases, e.g. firefly lucifera and bacterial lucifera (U.S. Patent No. 4,737,456), luciferin, 2,3-dihydrophthalazinediones, horseradish peroxidase (HRP), alkaline phosphatase,  $\beta$ -galactosidase, glucoamylase, lysozyme,  
30 saccharide oxidases, e.g. glucose oxidase, galactose oxidase, and glucose-6-phosphate dehydrogenase, heterocyclic oxidases such as uricase and xanthine oxidase, coupled with an enzyme that employs hydrogen peroxide to oxidize a dye precursor such as HRP, lactoperoxidase, or microperoxidase, biotin/avidin,  
35 spin labels, bacteriophage labels, stable free radicals, and the like.

Conventional methods are available to bind these labels covalently to proteins or polypeptides. For instance, coupling

agents such as dialdehydes, carbondiimides, dimaleimides, bisimidates, bisdiazotized benzidine, and the like may be used to tag antibodies with the above described fluorescent, chemiluminescent and enzyme labels. See for e.g. U.S. Patent  
5 Nos. 3,940,475 (fluorimetry) and 3,645,090 (enzymes); Hunter et al., *Nature*, 144:945 (1962); David et al., *Biochemistry*, 13:1014-1021 (1974); Pain et al., *J. Immunol. Methods*, 40:219-230 (1981); and Nygren, *J. Histochem. and Cytochem.*, 30:407-412 (1982). Preferred labels herein are enzymes such as  
10 horseradish peroxidase and alkaline phosphatase.

The conjugation of such label, including the enzymes, to the antibody is a standard manipulative procedure for one of ordinary skill in immunoassay techniques. See for e.g. O'Sullivan et al., "Methods for the Preparation of Enzyme-  
15 Antibody Conjugates for use in Enzyme Immunoassays", in *Methods in Enzymology*, Ed. J.J. Langone and H. VanVunkis, Vol. 73 (Academic Press, New York, New York, 1981), pp. 147-156.

Immobilization of reagents is required for certain assay methods. Immobilization entails separating the anti-pLDH  
20 antibody from any pLDH that remains free in solution. This conventionally is accomplished by either insolubilizing the anti-pLDH antibody or pLDH analogue before the assay procedure, as by adsorption to a water-insoluble matrix or surface (Bennich et al., U.S. Patent No. 3,720,760), by covalent  
25 coupling (for e.g., using glutaraldehyde cross-linking), or by insolubilizing the anti-pLDH antibody or pLDH analog afterward, e.g. by immunoprecipitation.

Other assay methods, known as competitive or sandwich assays, are well established and widely used in the commercial  
30 diagnostic industry.

Competitive assays rely on the ability of a tracer pLDH analog to compete with the test sample pLDH for a limited number of anti-pLDH antibody antigen-binding sites. The anti-pLDH antibody generally is insolubilized before or after the  
35 competition and then the tracer and pLDH bound to the anti-pLDH antibody are separated from the unbound tracer and pLDH. This separation is accomplished by decanting (where the binding partner was preinsolubilized) or by centrifuging (where the

binding partner was precipitated after the competitive reaction). The amount of test sample pLDH is inversely proportional to the amount of bound tracer as measured by the amount of marker substance. Dose-response curves with known amounts of pLDH are prepared and compared with the tests results to quantitatively determine the amount of pLDH present in the test sample. These assays are called ELISA systems when enzymes are used as the detectable markers.

Another species of competitive assay called a "homogenous" assay, does not require a phase separation. Here, a conjugate of an enzyme with the pLDH is prepared and used such that when aKushalnti-pLDH antibodies binds to the pLDH, the presence of the anti-pLDH antibody modifies the enzyme activity. In this case, the pLDH or its immunologically active fragments are conjugated with a bifunctional organic bridge to an enzyme such as a peroxidase. Conjugates are selected for use with anti-pLDH antibody so that binding of the anti-pLDH antibody inhibits or potentiates the enzyme activity of the label. This method per se is widely practiced under the name of EMIT.

Steric conjugates are used in steric hindrance methods or homogenous assays. These conjugates are synthesized by covalently linking a low-molecular-weight hapten to a small pLDH fragment so that antibody to hapten is substantially unable to bind the conjugate at the same time as the anti-pLDH antibody. Under this assay procedure, the pLDH present in the test sample will bind anti-pLDH antibody, thereby allowing anti-hapten to bind the conjugate, resulting in a change in the character of the conjugate hapten, e.g. a change in fluorescence when the hapten is a fluorophore.

Sandwich assays are also useful for the determination of pLDH or anti-pLDH antibodies. In sequential sandwich assays, an immobilized anti-pLDH antibody is used to adsorb test sample pLDH, the test sample is removed as by washing, the bound pLDH is used to adsorb a second, labeled anti-pLDH antibody and bound material is then separated from residual tracer. The amount of bound tracer is directly proportional to test sample pLDH. In "simultaneous" sandwich assays, the test sample is not separated before adding the labeled anti-pLDH. A

sequential sandwich assay using an anti-pLDH monoclonal antibody as one antibody and a polyclonal anti-pLDH antibody as the other is useful in testing samples for pLDH.

One type of assay which is particularly useful in testing samples for pLDH is the patented assay set forth in U.S. Pat. No. 5,124,141. This assay procedure is discussed in detail in the patent but briefly consists of the following. A blood sample, which may consist of whole blood, blood hemolysates, plasma, serum or the like, is combined with a specialized reagent. The reagent includes three main components. The first component is 3-acetyl pyridine adenine dinucleotide ("APAD") which functions as a coenzyme/necessary cofactor and is reduced enzymatically in the presence of pLDH. This material is commercially available from the Sigma Chemical Corporation of St. Louis, MO.

The second component is a substrate which is generally defined as a molecule whose chemical conversion is catalyzed by an enzyme. In a preferred embodiment, the substrate is a l-lactate salt..

The third component of the reagent is a buffer which is used to maintain the pH of the reagent at between about 8.0-10.0. Preferred buffers include AMPSO, CHES, bis-tris propane, AMP, and TRIS but other suitable buffers capable of maintaining the proper pH may also be used..

The reagent and blood sample are then combined. In a preferred sample, the reagent to blood volume ratio is about 1:300 and if serum is used the preferable range is from about 1:1 to about 1:20. If the blood sample contains any pLDH, the APAD is enzymatically reduced to APADH and the substrate is oxidized (e.g. lactate to pyruvate). The APADH may thereafter be detected using spectrophotometric techniques, colorimetrically, fluorimetrically, or electrophoretically. These detection methods are well known to those of ordinary skill in the art.

The foregoing are merely exemplary diagnostic assays for pLDH. Other methods now or hereafter developed that use anti-LDH antibody for the determination of pLDH are included within the scope hereof, including the bioassays described above.



All references cited in this specification are hereby expressly incorporated by reference. The following examples are offered by way of illustration and not by way of limitation. It should be appreciated that the *Plasmodium* used in each of the below examples are representative of all *Plasmodium* in that particular species since they each produce the same essential metabolic functions and characteristics.

All references cited in this specification are hereby expressly incorporated by reference. The following examples are offered by way of illustration and not by way of limitation.

#### Example 1

##### Production and Evaluation of Monoclonal pLDH Antibodies

The panel of anti-pLDH monoclonal antibodies was made by immunizing Balb-c mice to a protein fraction containing pLDH purified from red blood cells grown *in vitro* cultures of the *P. falciparum* strain D6. The authenticity of this source of pLDH was demonstrated by direct sequencing of the N-terminus.

For boosting immunization, recombinant pLDH was produced by subcloning a PCR fragment amplified from genomic DNA from the *P. falciparum* strain D6 into the expression vector pTrc99 (Pharmacia). *E. Coli* transformed with the resulting plasmid were subsequently induced with IPTG to produce recombinant pLDH (rpLDH). Recombinant pLDH is purified from bacterial lysates over a Cibacron blue sephrose and ion exchange column chromatography.

Purified recombinant pLDH was used to immunize a series of mice to raise monoclonal antibodies that specifically recognize pLDH. This data is summarized in Figures 1-4. Authentic pLDH was purified by Cibacron blue sepharose chromatography from culture lysates (Fig. 1). pLDH activity (ADAP) can be separated from human red blood cell LDH (1st NAD peak) because pLDH binds tightly to Cibacron blue. Recombinant pLDH produced in bacteria is purified the same way and shows identical biochemical characteristics to authentic pLDH.

Hybridoma cell lines were subcloned until monoclonality was established. Antibodies were harvested from serum free

media and purified by the sequential precipitation with ammonium sulfate and caprylic acid. Antibodies were subtyped as:

- 19G7-C10; IgG1,  $\kappa$
- 5 7G9-G1; IgG1,  $\kappa$
- 17E4-D6; IgG1,  $\kappa$
- 6C9(A)-D6; IgG1,  $\kappa$
- 6C9(B)-G7; IgA,  $\kappa$

Figure 2 shows an agarose electrophoresis gel in which active enzyme has been stained with NAD<sup>+</sup>, NBT and PES. Active recombinant pLDH was used as an immunogen to raise the monoclonal antibodies, 19G7, 7G9, 17E4, 6C9a, and 6C9b.

These antibodies have been used to coat microtiter plates (see Figure 3). These coated plates are capable of capturing enzymatically active pLDH from samples of whole blood, plasma, or lysed red blood cells. The immuno-enzyme capture assay (ICpLDH) is able to capture small amounts of pLDH from blood products and allow for the unambiguous measurement of pLDH activity.

Four of the MAbs captured active enzyme without adverse effects on enzyme activity. In contrast, the 6C9a antibody captured pLDH but at high concentrations inhibits enzyme activity. All five antibodies were subcloned and prepared and purified on a large scale.

25

#### **Formatting and Testing of a Solution Based Immunocapture pLDH Assay**

To insure that the pLDH assay would reflect % parasitemia in patient samples, the specific activity of pLDH must be similar in different isolates. Therefore, the specific activity of pLDH from different patient samples was measured. Samples of high parasitemia were allowed to bind to coated microtiter plates such that the enzyme was in excess. Since all of the antibody sites were then occupied by pLDH, the relative specific activities could be measured. This was done in triplicate for seven samples of *P. falciparum* and three samples of *P. vivax*. All *P. falciparum* samples had similar activities (see Fig. 4).

Tabular data is presented for a subset of data generated from two studies. For each study, patient samples (100 µls) were incubated in microliter wells for 40 minutes after which wells were washed with PBS. In the first studies, wells were allowed to react with 3-acetyl pyridine adenine dinucleotide (APAD) for 20 minutes and read at 365 nm after which a solution of NBT and Diaphorase was added to assay for pLDH activity colorimetrically. These experiments gave reproducible results, however, this assay was non linear with respect to enzyme activity and required modification. This included the addition of APAD and NBT/Diaphorase. The kinetics of this later reaction were linear as a function of enzyme concentration. A standard curve showing the sensitivity of this ICpLDH assay is shown in Figure 5.

A starting parasitemia of 10% was diluted in whole uninfected blood to yield samples of the indicated % parasitemia. Either 100 µls or 5 µls of the sample were incubated in coated microtiter wells for 30 minutes. The sample volumes were adjusted to 150 µls total with the addition of PBS containing 1% BSA. The wells were previously coated with either the 19G7 monoclonal antibody (pan-specific) or the 17E4 monoclonal antibody (*P. falciparum* specific). the wells were then washed 2 times with PBS and incubated with APAD containing NBT and Diaphorase. The microtiter wells are shown in Figure 6.

#### **The Specificity of the New PLDH Amino capture assay (ICtLDH)**

Preliminary findings reveal that MABs 19G7, 6C9a and 6C9b are able to capture both *P. falciparum* and *P. vivax* LDH, while two other antibodies, 17E4 and 7G9 capture only pLDH from *P. falciparum*. *P. vivax* pLDH blood samples from Central America, West and South Africa, India and Papua New Guinea (Figure 7) all perform as described with the MAB reagents. A visual depiction of the results is set forth in Figure 8. In all cases, 19G7, 6C9a and 6C9b MABs were able to capture active *P. vivax* pLDH. All five antibodies were able to capture *P. falciparum* pLDH. This result shows that there are epitopes both common to pLDH from different species as well as unique

species specific epitopes. Because some antibodies recognized unique epitopes of *P. falciparum* and *P. vivax* pLDH, the reactivity of these antibodies to other *Plasmodium* species were tested. In general, the 19G7 and 6C9a antibodies recognized a wide range of *Plasmodium* LDH isoforms. In particular, 6C9a recognized all isoforms tested. Based on previous data on other *Plasmodium* enzymes, it is fully expected that the 6C9a antibody will be fully capable of recognizing the pLDH isoforms in these species as well.

#### Clinical Studies

In the first study, over 135 samples from 75 patients were tested. Several serial samples from a subset of patients were evaluated in the study. These samples were evaluated by microscopic inspection of Giemsa thin smears. The first study was restricted to samples infected with *P. falciparum*. This data is presented in Table 1. The pLDH assay did not yield any false negatives. Besides the nineteen samples judged parasite free by microscopy, noninfected specimens of whole blood from 35 other patients with a wide range of ailments were also analyzed. None of these samples were positive by ICpLDH assay. The pLDH assay was able to detect all patients samples with parasitemias of *P. falciparum* of  $> 0.06\%$  ( $> 3,000 \mu\text{l}$ ). Within the range of  $0.001\text{--}0.05\%$ , the pLDH test was positive with  $90\%$  of the samples. Curiously, three of the false negative samples were from a single patient (Patient #64). As with all of these data points, the samples were part of a series taken from a patient undergoing antimalarial treatment. The initial sample taken was positive by ICpLDH assay, demonstrating that the reason for false negative result was not due to a lack of antibody specificity.

Further analysis of these samples by SDS-PAGE and immunoblot showed that the immunoreactive material was degraded and thus provided one explanation for the loss of pLDH activity. A second explanation is that treatment destroyed the parasites and thus the pLDH activity. Further studies are needed to validate these explanations. Elimination of this patient's samples from the analysis would bring the sensitivity of the pLDH assay to  $\sim 97\%$  for samples of  $0.001\text{--}0.05\%$ . The

ICpLDH assay was able to detect ~83% of samples with parasitemias of <0.001 (<50/ $\mu$ l).

Tabl 1.

Immunocapture test with *P. falciparum* Infected Blood

5

<u>Giemsa</u>		<u>pLDH Immunocapture Activity Assay</u>		
% Parasitemia	Parasites/ $\mu$ l	Positive	Negative	Sensitivity
>0.06	3,000	45	0	100%
0.06-0.001	50-3,000	39	4 (1*)	90% (97%)
10 >0.001	<50 or gametocytes	25	5 (4*)	83% (87%)
				Specificity
control	0	0	19	100%
Total				
137 Samples				
75 Patients				

20 Most of the samples tested in the first study presented above were from patients infected with malaria in India and Africa. In another study, samples from 26 patients collected in Central America, Cali, Columbia were evaluated. The range of parasitemias tested were from 42 - 15,000 parasites/ $\mu$ l

25 (0.001-0.3% parasitemia). Patient samples infected with either *P. vivax* or *P. falciparum* were evaluated in one of two ways: Samples were either stored on ice until evaluation or were absorbed to sheets of Whatman 3M paper, dried and stored at room temperature. To assay the dried samples, a 1 cm<sup>2</sup> are of

30 the paper was soaked in 300  $\mu$ ls of PBS for 20 minutes. 200  $\mu$ ls of this solution was used in the ICpLDH test with the 19G7 antibody. The results from these studies are presented in Table 2. In all cases, the ICpLDH assay was able to identify samples from patients infected with either *P. falciparum* or *P.*

35 *vivax*. This was true for whole blood samples stored at 4°C and for samples absorbed to Whatman 3M paper and stored at room temperature for 7 months. Also, no false positives were detected by the ICpLDH assay. To further support this finding, uninfected control blood from 10 different sources was dried on

40 Whatman paper and subjected to extraction and the ICpLDH test.

No false positives were detected with these additional samples (data not shown).

Table 2 shows the efficacy of the diagnostic test for the detection of *P. vivax*. Results from samples collected at  
 5 Portland Veterans Administration Hospital, the London Hospital for Tropical Disease, and from a collaborative clinic in Cali Columbia were combined and tabulated.

In the most extensive evaluation of *P. falciparum* samples, it was found that within the range the ICpLDH assay was 96%  
 10 sensitive and 100% specific for samples of >0.001% parasitemia. These studies show that the ICpLDH is well suited for diagnosis of malaria. By using a rapid purification procedure based on the immobilization of active pLDH in microliter wells, false  
 15 positives are eliminated and the sensitivity of the pLDH assay is increased. The use of the 19G7 antibody also affords the detection of both *P. vivax* and *P. falciparum*. Further, it has been shown that other anti-pLDH monoclonal antibodies are specific for pLDH from *P. falciparum*. Therefore a simple test  
 20 could be formatted to speciate *Plasmodium* infections.

Table 2.  
 Immunocapture test infected Blood: South America

25

		pLDH Immunocapture Activity				<u>Giemsa</u>
		<u>Assay</u>				
		4°C storage		Dry Storage		
%	Parasites sp.	Positive	Negative	Positive	Negative	
0.25-0.001	<i>P. falciparum</i>	8	0	8	0	
0.8-0.004	<i>P. vivax</i>	12	0	12	0	
0	Control	0	5	0	5	
Total						
25 samples						
25 patients						

30

One important consideration which must be reemphasized is that, in interpreting the above data, one must remember that the apparent sensitivity of the pLDH test was ascertained by testing samples from patients undergoing anti malarial therapy.  
 35 Thus, the starting parasitemias of these patients were often quite high (0.01-1%). All of the low parasitemia samples were from patients that had been treated for at least 2-3 days.

Therefore, the viability of the parasites within these samples may have been severely compromised. pLDH activity is an indicator of viable parasites. Consequently, the samples that were originally designated with "low parasitemia" or no parasitemia may in fact be samples that contain non viable parasites.

On important aspect of the ICpLDH assay is apparent from inspection of several samples of patients undergoing anti malarial treatment. These data sets show that pLDH activity presents in peripheral red blood cells drops in concert with parasitemia. It is possible that plasma levels of pLDH may reflect total parasite load (sequestered and peripheral parasites).

#### Comparison with ParaSightF™

ParasightF™ antigen detection assay was found to perform comparably to the assay of the present invention using MABs. Within a very low range of parasitemias ( $<0.001\%$  or 50  $\mu\text{ls}/\mu\text{l}$ ) some infected samples were found positive by pLDH but not by ParasightF™ as well as vice versa. Upon analyzing serial samples from patients undergoing anti malarial chemotherapy, the ParasightF™ assay, in contrast to the ICpLDH assay and the standard thin smear, consistently gave a positive signal even when parasites were completely cleared as judged by microscopy. This persistent antigenemia was readily apparent in some patients who were tested 14 days after antimalarial treatment, 10 days after they were judged parasite free by microscopy. This data again underscores the utility of the pLDH assay. The fact that active pLDH levels do not persist once parasites have been killed combined with the quantitative aspect of the ICpLDH assay make it possible to use the present test to monitor patient progress with a given antimalarial chemotherapy regimen. This ability is a major requirement for identifying and assessing drug resistant infection in vivo. Preliminary studies with a dry "dip stick" antigen detection based assay for pLDH also indicate that the presence of pLDH antigen also does not persist after parasite clearance.

Formatting of a Dry Immuno-Capture pLDH Assay

5 A nitrocellulose strip was coated with a line of 17E4  
monoclonal antibody (*P. falciparum* specific) and a second line  
of 19G7 monoclonal antibody (pan specific). The blood sample  
was then lysed with a bead conjugate made with the 6C9a  
antibody. Thus, each of the antibodies used had a separate  
epitope. The lysate was then wicked up the nitrocellulose  
strip. If the sample contained pLDH (from *P. falciparum*) the  
bead conjugate (with bound pLDH) would bind to the 17E4 line.  
10 If the bead conjugate has pLDH from *P. vivax* bound, the  
conjugate would pass over the 17E4 line and bind only to the  
19G7 line. It is anticipated that the dip stick will be able  
to differentiate between *P. vivax* and *P. falciparum* and is  
capable of detecting 5 viable parasites/ $\mu$ l of blood, thus  
15 providing a solid phase "dip-stick" assay.

The above description thus sets forth a novel and simple  
to use diagnostic assay for malaria based on the quantitation  
of pLDH. It further demonstrates a novel series of monoclonal  
antibodies which are useful in the speciation of *Plasmodium*  
20 isoforms in order to determine which particular is causing the  
infection in the host. It is therefore submitted that the  
present invention accomplishes at least all of its stated  
objectives.



What is claimed is:

1. A diagnostic method for detecting the presence of viable *Plasmodium* species in animals comprising: obtaining a sample  
5 of body fluid from said animal; and assaying for the presence of lactate dehydrogenase with a monoclonal antibody which do s not cross react with lactate dehydrogenase from humans, bacteria, or other parasites.
- 10 2. The diagnostic method according to claim 1 wherein the body fluid is a blood sample.
3. The diagnostic method according to claim 2 wherein the blood sample is selected from the group consisting of whole  
15 blood, hemolyzed red blood cells, plasma, and serum.
4. The diagnostic method according to claim 1 wherein said monoclonal antibody is labeled for detection.
- 20 5. The diagnostic method according to claim 1 wherein said monoclonal antibody reacts to form an antibody/antigen complex with *Plasmodium* lactate dehydrogenase.
6. The diagnostic method according to claim 5 wherein said  
25 step of assaying includes: detecting the presence of said antibody/antigen complex using a method selected from the group consisting of spectrophotometrically, colorimetrically, and fluorometrically.
- 30 7. A diagnostic method for detecting the presence of viabl *Plasmodium* species in animals comprising: obtaining a body fluid sample; combining the sample with an enzyme activity assay reagent and a monoclonal antibody wherein the monoclonal antibody captures an enzyme specific to *Plasmodium* species and  
35 the reagent becomes reduced; and detecting the presence of the reduced reagent.

8. A diagnostic method according to claim 7 wherein the enzyme is *Plasmodium* lactate dehydrogenase.
9. A diagnostic method according to claim 7 wherein the enzyme activity assay reagent is comprised of: 3-acetyl pyridine adenine dinucleotide; a substrate; and a buffer.
10. A diagnostic method according to claim 7 wherein the body fluid is selected from the group consisting of whole blood, hemolyzed red blood cells, plasma, serum, and saliva.
11. A diagnostic method according to claim 7 wherein the body fluid sample is captured with the monoclonal antibody and the enzyme activity assay reagent in a volume ratio of from about 1:1 to 1:300.
12. A diagnostic method according to claim 9 wherein the substrate is a lactate salt or lactic acid.
13. The diagnostic method according to claim 7 wherein the *Plasmodium* species is selected from the group consisting of *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovalae*, and *Plasmodium malariae*.
14. The diagnostic method according to claim 7 wherein said step of assaying includes: quantifying the *Plasmodium* lactate dehydrogenase by a second immobilized monoclonal antibody which specifically binds *Plasmodium* lactate dehydrogenase at a distinct, non overlapping epitope from the first antibody.
15. The diagnostic method of claim 14 wherein one of said monoclonal antibodies is labeled for detection.
16. The method of claim 14 wherein the monoclonal antibody is present in a "wet" enzymatic capture assay capable of detecting malarial infections of a peripheral parasitemia of  $\leq 0.001\%$ .

17. A diagnostic method according to claim 16 wherein the "wet" enzymatic capture assay may be used with a body fluid sample comprising whole blood or red blood cells wherein the whole blood or red blood cells may be fresh, frozen, or absorbed and dried onto filter paper.
18. A diagnostic method according to claim 14 wherein the monoclonal antibody is formatted into a "dry" wick-stick antigen detection strip.
19. A diagnostic method according to claim 18 wherein *Plasmodium* lactate dehydrogenase is visualized using antibody conjugates selected from the group consisting of colloidal gold, latex beads, and colorimetric dyes.
20. A diagnostic method according to claim 18 wherein the "dry" wick-stick antigen detection strip can be used to differentiate between different *Plasmodium* species that cause malaria in humans.
21. A monoclonal antibody which specifically captures *Plasmodium* lactate dehydrogenase wherein said monoclonal antibody is selected from the group consisting of 19G7-C10, 7G9-G1, 17E4-D6, 6C9(A)-D6, and 6C9(B)-G7.
22. A monoclonal antibody according to claim 21 wherein said monoclonal antibody does not cross react with lactate dehydrogenase isoforms present in the whole blood of other animal, bacterial, or parasitic species.
23. A series of monoclonal antibodies according to claim 21 wherein the monoclonal antibodies bind to distinct non overlapping epitopes.
24. A monoclonal antibody according to claim 21 wherein said monoclonal antibody can distinguish between different human and non-human species of *Plasmodium* which cause human malaria.

25. A monoclonal antibody according to claim 21 wherein said monoclonal antibody can capture *Plasmodium* lactate dehydrogenase in the fully active form.

5 26. A monoclonal antibody according to claim 21 wherein said monoclonal specifically binds lactate dehydrogenase from *Plasmodium vivax*, *Plasmodium ovalae*, and *Plasmodium malariae* and is selected from the group consisting of 19G7-C10, 6C9(A)-D6, 6C9(B)-G7.

10 27. An immunocapture test capable of detecting human malarial infections comprising: a monoclonal antibody which captures *Plasmodium* lactate dehydrogenase; an immunoreactive agent capable of detecting bound antibody antigen complexes and said  
15 agent providing for detection of the presence of *Plasmodium* lactate dehydrogenase in body fluid.

20 28. An immunocapture test according to claim 27 wherein the body fluid is a blood sample comprising whole blood or red blood cells.

25 29. An immunocapture test according to claim 28 wherein the blood sample is selected from the group consisting of fresh, frozen, and absorbed and dried on filter paper.

30 30. An immunocapture test according to claim 27 wherein the test can be formatted with a standard curve using recombinant *Plasmodium* lactate dehydrogenase for quantitation of *Plasmodium* lactate dehydrogenase activity.

35 31. An immunocapture test according to claim 30 wherein the quantitation of *Plasmodium* lactate dehydrogenase activity from body fluid samples can be used to monitor patient response to antimalarial treatment.

32. An immunocapture test according to claim 27 wherein the test can be used on large batches of blood samples that are encountered in blood banking and epidemiological surveys.

33. An immunocapture test according to claim 30 wherein the quantitation of *Plasmodium* lactate dehydrogenase activity in body fluid samples can be used to evaluate the effect of anti-malarial drugs *in vitro* and *in vivo*.

34. An immunocapture test according to claim 27 wherein the monoclonal antibodies are selected from the group consisting of 19G7-C10, 7G9-G1, 17E4-D6, 6C9(A)-D6, and 6C9(B)-G7.

35. An immunocapture test according to claim 27 wherein the 19G7-C10, the 6C9(A)-D6, and the 6C9(B)-G7 antibodies are capable of capturing *Plasmodium* lactate dehydrogenase from *Plasmodium* species selected from the group consisting of *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovalae*, and *Plasmodium malariae*.

36. An immunocapture test according to claim 34 wherein the 6C9(A)-D6, 6C9(B)-G7 and 19G7-C10 monoclonal antibodies are capable of capturing *Plasmodium* lactate dehydrogenase from non-human *Plasmodium* species.

37. A method for detecting malaria in humans comprising: obtaining a blood sample suspected of containing malaria-causing *Plasmodium* from a human; and assaying for the presence of *Plasmodium*-lactate dehydrogenase in said sample with a monoclonal antibody which specifically binds *Plasmodium* lactate dehydrogenase and which does not cross react with lactate dehydrogenase from humans, bacterial, parasitic or other species.

38. A monoclonal antibody for detecting malaria in humans which reacts essentially with *Plasmodium* lactate dehydrogenase but not with lactate dehydrogenase isoforms present in the body fluids of other animal, bacterial, or parasitic species.

39. The monoclonal antibody of claim 38 wherein the monoclonal antibody is selected from the group consisting of 19G7-C10, 7G9-G1, 17E4-D6, 6C9(A)-D6, and 6C9(B)-G7.

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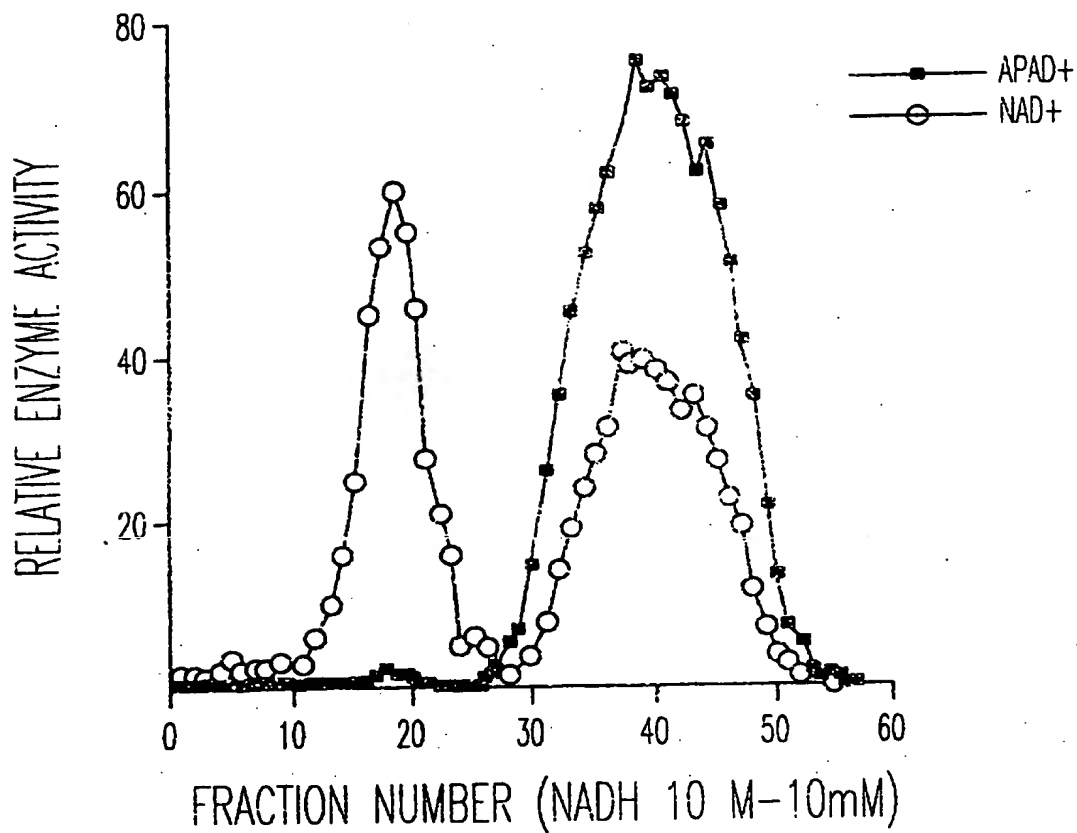


Fig. 1

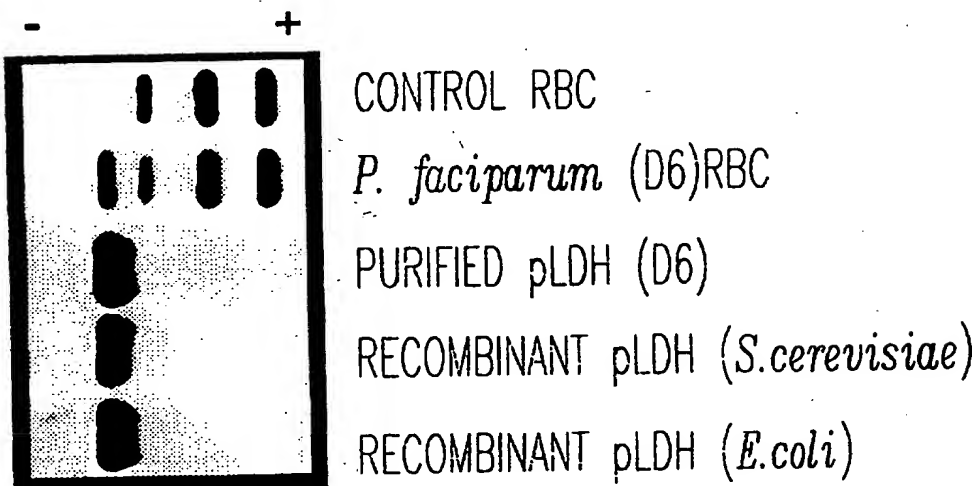


Fig. 2





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## ANTIBODY

19G7 7G9 17E4 6C9a 6C9b

*P. vivax* (S. AMERICA)*P. falciparum* (ZIMBABWE)*P. falciparum* (GAMBIA)*P. vivax* (INDIA)

CONTROL

RECOMBINANT pLDH

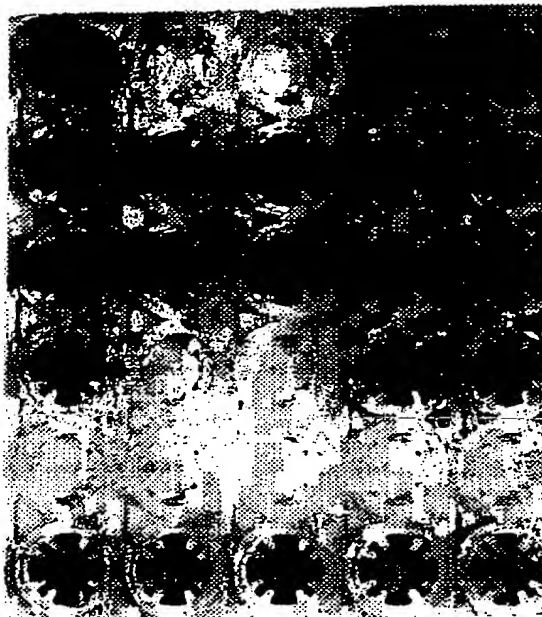


Fig. 3

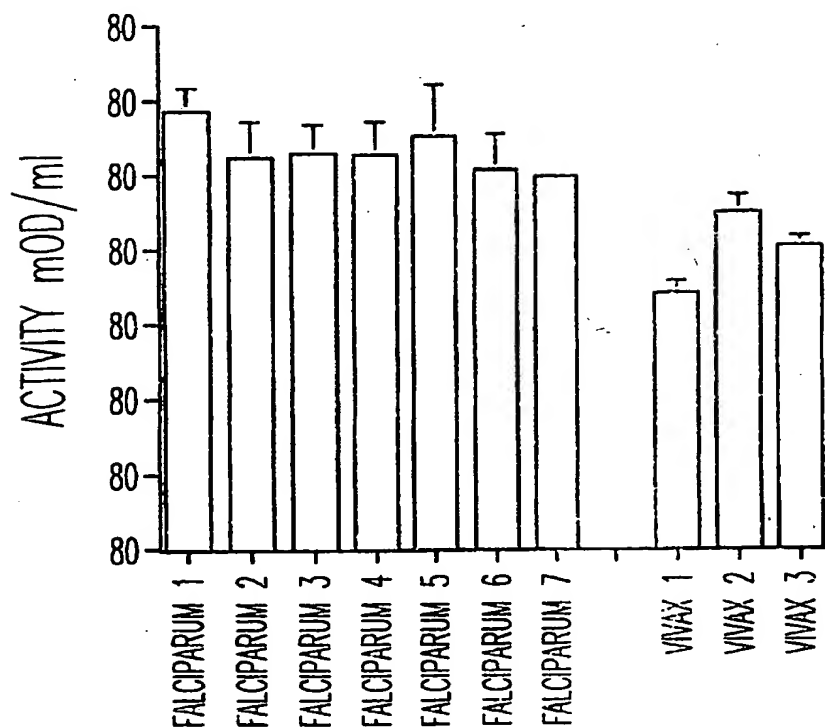


Fig. 4



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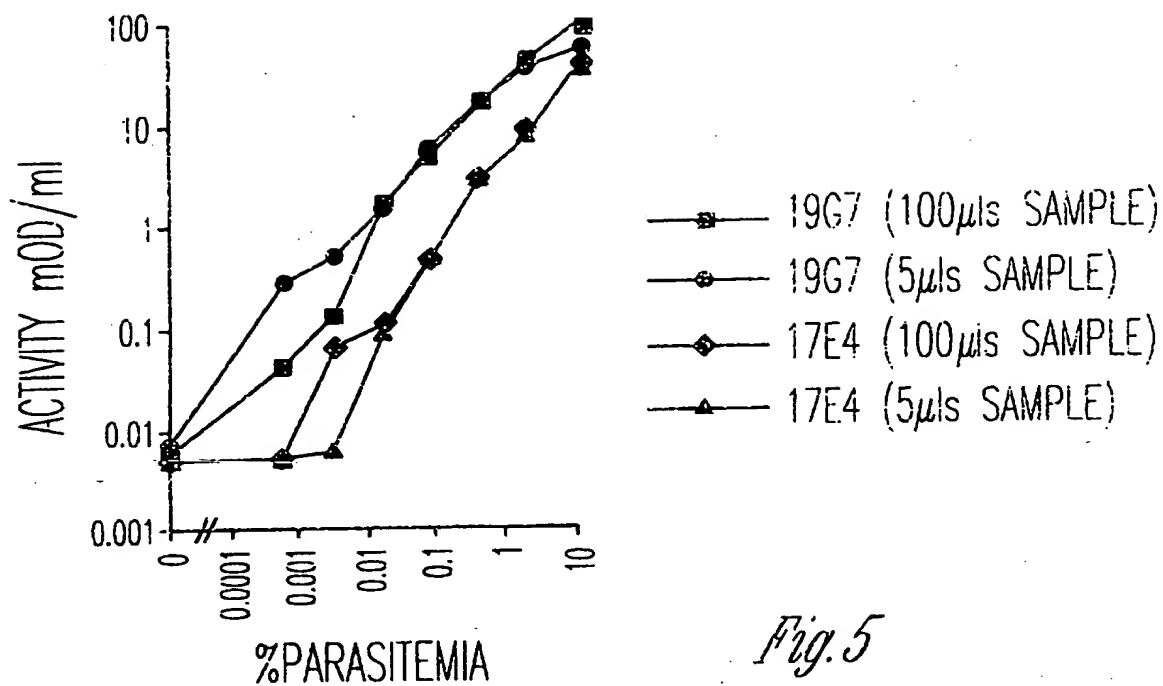


Fig. 5

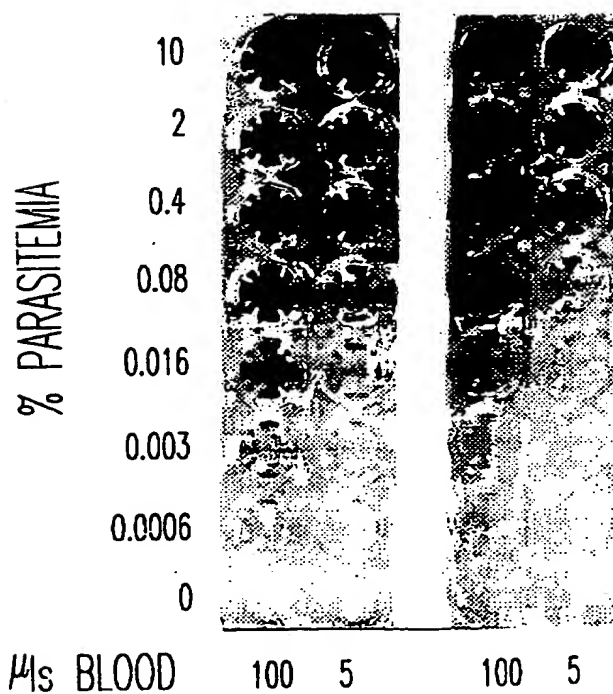
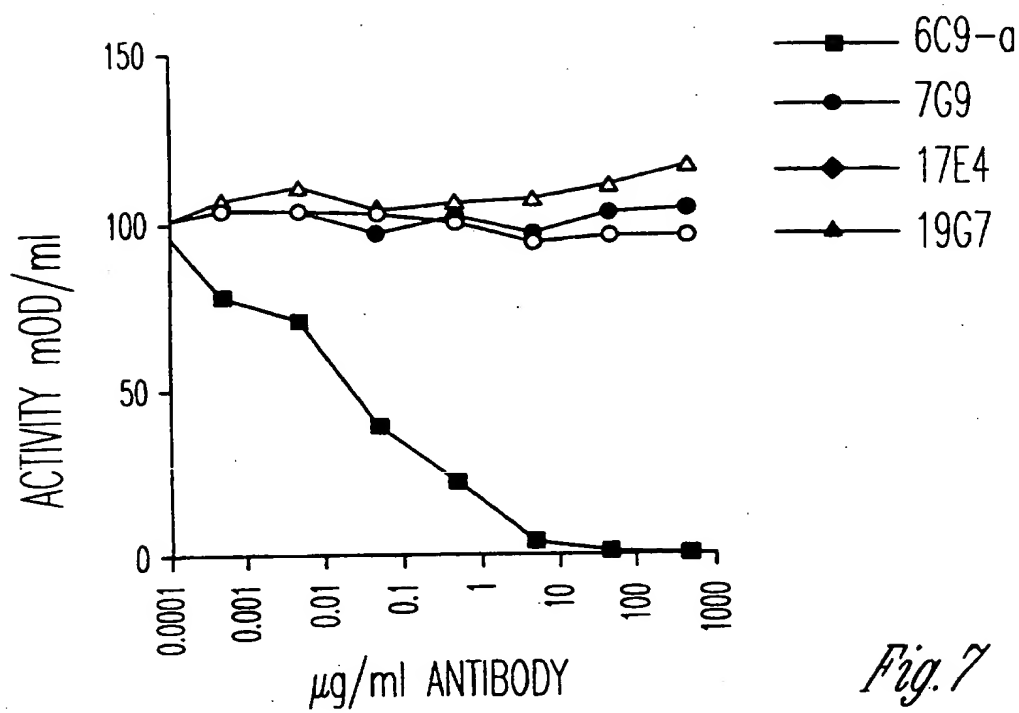


Fig. 6



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*Fig. 7*



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7G9 17E4 19G7 6C9b 6C9a

					<i>P. falciparum</i> (1)
					<i>P. falciparum</i> (2)
					<i>P. falciparum</i> (3)
					<i>P. falciparum</i> (4)
					<i>P. vivax</i> (1)
					<i>P. vivax</i> (2)
					<i>P. vivax</i> (3)
					<i>P. vivax</i> (4)
					<i>P. vivax</i> (AOTUS)
					<i>P. cynomogli</i>
					<i>P. berghel</i>
					<i>P. vincke</i>
					<i>P. gallinaceum</i>
					<i>P. retictum</i>
					<i>Bovis babesia</i>
					HUMAN CONTROL
					SIMIAN CONTROL
					MURINE CONTROL
					AVIAN CONTROL
					BOVIS CONTROL

Fig. 8





## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/20672

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : Please See Extra Sheet.

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/130.1, 141.1, 146.1, 151.1; 435/4, 7.1, 7.2, 7.4, 7.9, 7.92, 7.93, 7.94; 530/387.1, 391.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAplus, Medline, Biosis, EMBASE, SciSearch, WPIDS, APS

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	WO 94/24287 (TRUSTEES OF DARTMOUTH COLLEGE) 27 October 1994, See especially Abstract.	1-6, 27-33, 37-38 ----- 21-27, 30-36, 38,38
Y	MAKLER MT et al. Laboratory Diagnosis of Malaria. Clinics in Laboratory Medicine. December 1991. Vol. 11, No. 4. pages 941-956.	7-17
Y	UGUEN C et al. ParaSight-F rapid manual diagnostic test of Plasmodium falciparum infection. Bulletin of the World Health Organization. 1995. Vol. 73. No. 5. pages 643-649.	7, 14, 18-20

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	* T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
* A* document defining the general state of the art which is not considered to be of particular relevance	* X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
* E* earlier document published on or after the international filing date	* Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
* L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	* A*	document member of the same patent family
* O* document referring to an oral disclosure, use, exhibition or other means		
* P* document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

29 MARCH 1997

Date of mailing of the international search report

24 APR 1997

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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/20672

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SHIFF CJ et al. The ParaSight-F test: A Simple Rapid Manual Dipstick Test to Detect Plasmodium falciparum Infection. Parasitology Today. 1994. Vol. 10. No. 12. pages 494-495.	7, 14, 18-20
Y	THORPE SJ et al. Immunochemistry: Lab Fax. UK: Bios Scientific Publishers. 1994, pages 175-177, especially page 177.	27, 30-33
Y	US 5,124,141 A (MAKLER) 23 June 1992, Abstract and column 8.	7-17
Y	SEVIER ED et al. Monoclonal Antibodies in Clinical Immunology. Clin. Chem. 1981. Vol. 27. No. 11. Pages 1797-1806	21-26, 34-36, 38, 39
A	MAKLER MT et al. Measurement of the Lactate Dehydrogenase Activity of Plasmodium falciparum as an Assessment of Parasitemia. 1993. Vol. 48. No. 2. pages 205-210. See especially the entire document.	1-39

Form PCT/ISA/210 (continuation of second sheet)(July 1992)\*

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/20672

## A. CLASSIFICATION OF SUBJECT MATTER:

IPC (6):

A61K 39/395; C07K 16/00; C12Q 1/00, 1/04; G01N 33/53, 33/569, 33/573

## A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

424/130.1, 141.1, 146.1, 151.1; 435/4, 7.1, 7.2, 7.4, 7.9, 7.92, 7.93, 7.94; 530/387.1, 391.1

